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Award Number: W81XWH-08-1-0084

TITLE: Using Human Stem Cells to Study the Role of the Stroma in the Initiation of Prostate Cancer

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REPORT DATE: March 2011

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                      Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-03-2011	Final	1 MAR 2008 - 28 FEB 2011

4. TITLE AND SUBTITLE

Using Human Stem Cells to Study the Role of the Stroma in the Initiation of Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER

W81XWH-08-1-0084

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Monash University
Clayton, Victoria 3168 AUSTRALIA

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Abstract on next page.

15. SUBJECT TERMS

Not provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU	35	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

14. ABSTRACT

This project aimed to determine the role of tumor stroma in prostate cancer biology. To do this, we used a model of human embryonic stem cell (hESC) differentiation that was established in our laboratory. Using hESC-derived prostatic epithelial cells, we attempted to determine whether or not tumor stroma derived from human prostate cancer specimens induce and initiate carcinogenesis.

The data generated in this funded project failed to support the hypothesis that prostatic tumour stroma was an initiator of tumorigenesis, but rather findings indicated that preceding events in the epithelial cells are most likely required to begin the process of malignancy. We went on to prove that CAF-induced malignancy was restricted to intermediate/transient amplifying (CD133-) cells, but not stem (CD133+) cells under the influence of stroma. This is an important finding, and provides new information regarding the initiation of prostate cancer, and the potential cancer cell of origin.

Defining the role of prostatic tumour stroma in the initiation of carcinogenesis significantly impacts on the field of prostate cancer (and other major cancers). These findings, based on an innovative approach using human prostate stromal cells and embryonic stem cells, have provided fundamental advances to our understanding how cancer is initiated and thus may be prevented or treated. Our current data implicate both transient amplifying epithelial cells and stromal components of the tumor as therapeutic targets.

Risbridger, Gail P.   Award: W81XWH-08-1-0084   Final Report March 2011

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Introduction

The role of tumor stroma in prostate cancer biology is equivocal. Current dogma suggests that prostate carcinogenesis is a multi-step process involving genetic alterations in the epithelium that drives the progressive transformation of normal human cells into highly malignant derivatives. It is evident that tumor stroma is able to promote progression of tumorigenesis, but whether it also plays a critical role in the initiation of tumor formation is unclear (*1*).

Epithelial cells are under the control of the underlying mesenchymal cells during embryogenesis and throughout life (*2*); it is therefore our **hypothesis that the prostatic stromal cells have the capacity to initiate carcinogenesis in normal epithelial cells**. In order to address the issue of tumor initiation, we propose to use normal human prostate epithelium generated from human embryonic stem cells (hESCs) in tissue recombination studies with tumor stroma from human prostate cancer patients.

In this project, we propose to use hESCs as a source of normal human prostate epithelial cells. Normal human prostate tissue from adult men in the prime of his life is difficult to obtain, and human fetal tissue is of limited availability. We successfully achieved this goal and published the findings in Nature Methods (*3*).

Body

Task 1 (Aim 1.1): _To improve our current method of directing hESC differentiation to obtain genetically normal human prostatic epithelial cells_ [Years 0-1.5].

   a. Culture and maintenance of human embryonic stem cells (hESCs); including routine karyotyping and identification of other pluripotent markers of undifferentiated hESCs.

During the funded period, a core facility was established at Monash University to produce hESCs. Therefore, we no longer needed to spend time or effort in maintaining our own colonies of hESCs, which is technically challenging. We obtained organ culture dishes of hESCs to pre-differentiate into endoderm, or use for recombination experiments. All routine karyotyping and identification of pluripotent cell markers was taken care of by the core research staff. We can confirm that our source of hESCs was maintained to a high standard, by a facility that produces cells and ships them out to research laboratories around the world. We routinely used two hESC lines for this research, specifically hES3 cells, and a genetic variant of that line, ENVY, which expresses GFP for tracing cells.

   b. Pre-differentiation of hESCs using 100ng/ml activin A in serum free conditions for 5-8 days into endoderm _in vitro_. Confirm endoderm phenotype using immunohistochemistry and FACs analysis.

We conducted these experimental procedures as outlined in the research plan, previously published by D'Amour and colleagues (_4, 5_). This challenging procedure produced variable results. On average, we generated a cell population of ~60-80% definitive endoderm cells from hESCs using activin A. Dual fluorescent labeling with Sox17 and CXCR4 were used to qualitatively determine the % endoderm in differentiated samples, although an exact quantitation of samples used for recombination could not be determined, since cells cannot be used for histology and recombination in parallel. Nonetheless, we collected a separate aliquot of pre-differentiated cells for analysis, and only used samples of >60% Sox17/CXCR4-positive immunostaining. FACs analysis was used on a few occasions, as we experienced difficulties in generating sufficient cell numbers that were Sox17/CXCR4-positive and viable following staining and flow cytometry for use in recombination experiments.

   c. Generation of tissue recombinants of endoderm-derived hESCs together with rodent UGM or SVM (isolated from E17.5 male embryos for UGM or day 0 male pups for SVM) using collagen gel technique and sub-renal grafting into male SCID mice.

We completed a series of tissue recombination experiments, using undifferentiated and pre-differentiated (activin A-treated) hESCs. This allowed us to test whether using hESCs that were pre-differentiated to endoderm derivatives could generate a higher efficiency of prostatic structures, compared to undifferentiated hESCs that were reported in our publication (_3_).

There were three experimental groups analyzed, all using rat urogental mesenchyme (rUGM) and ENVY cells:

1. rUGM + undifferentiated hESCs
2. rUGM + activin A-treated hESCs
3. hESCs alone (either undifferentiated or activin-A treated)

    d.   Harvesting and analysis of tissue recombinants including immunohistochemistry for morphological analysis and cell death/proliferation markers.

In each experimental group, at least n=5 grafts were analyzed, but as many as n=12 in some groups. The figures presented below show representative data in terms of graft size, survival of hESCs in tissue recombinants, and % of glandular epithelial cells that express androgen receptor (AR) as an indicator of prostatic structures. We used staining for PSA (prostate specific antigen) to confirm these ducts were prostatic, and not other male reproductive tract structures, and in all cases, both PSA and AR co-localized.
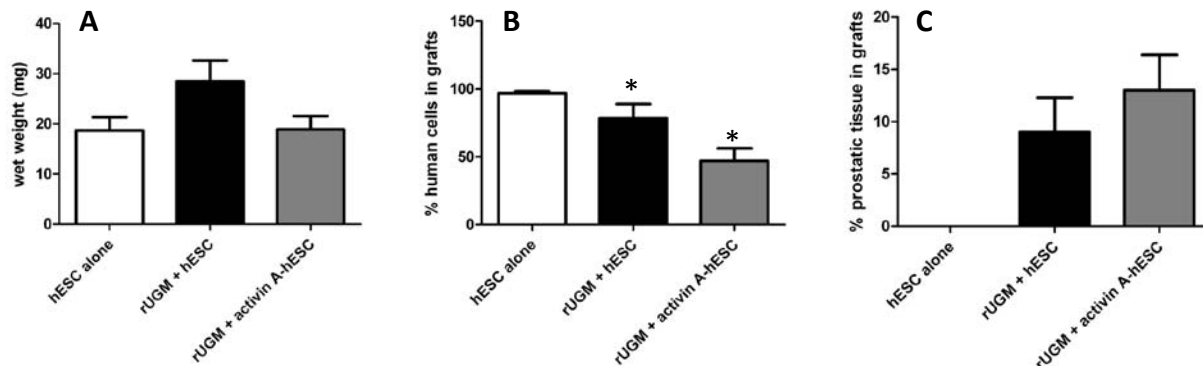
Figure 1: Pre-differentiation to endoderm does not increase efficacy of generating prostatic epithelia. (**A-C**) Tissue recombinants of either hESCs alone, hESCs with rat UGM or pre-differentiated-hESCS with rat UGM. Data are for graft wet weight (**A**), % human tissues in graft (distinguishing from contribution from rat UGM or mouse host cells (**B**) and % prostatic tissue in grafts as determined by PSA and androgen receptor localization (**C**). Data are mean ± S.E.M, p <0.05, one way ANOVA with Tukey post-hoc test).

There was no difference in the growth or survival of hESCs whether they were pre-differentiated into endoderm or not (**Figure 1**). We did see a great proportion of the grafts exhibiting endoderm-derived structures in using pre-differentiated hESCs, but the increase in prostatic tissue observed was minor. Although the hESCs were encouraged to be directed down a more direct differentiation path, non-prostatic endoderm structures spontaneously arose. Nonetheless, prostate-like glands, pathologically similar to our original publication (*3*) were observed (**Figure 2**). Based on these findings, we went on to conduct experiments described in the alternative method below.
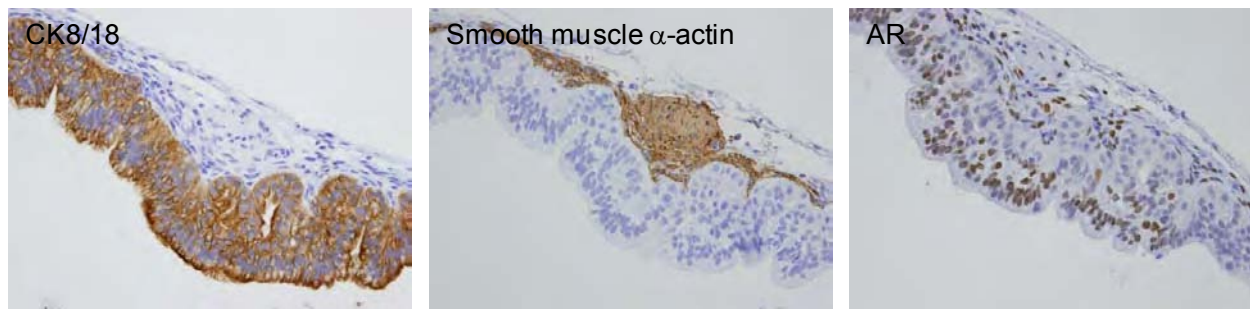
Figure 2: hESC cells differentiated into prostate-like glands with rat UGM. Expression of cytokeratins 18/18 for (human-specific) epithelial cells, smooth muscle α-actin in surrounding stromal cells, and androgen receptor (AR) localization in prostate-like glandular epithelium and surrounding stromal cells.

       e.   <u>ALTERNATIVE METHOD</u>: perform two-step tissue recombination with endoderm-derived hESCs and rodent UGM or SVM using collagen gel technique and sub-renal grafting into male SCID mice, if first method is not optimal.

To do this, we harvested tissue recombinants composed of rUGM + undifferentiated hESC or rUGM + pre-differentiated hESCs. As stated above, the wet weights of the harvested tissues were not difference between the groups, and each graft was cut into 4 separate tissue pieces and recombined with further rUGM in a second round of tissue recombination. After 8 weeks in host SCID mice, we harvested the tissues. Using morphology and immunostaining analysis, we showed that the % of prostatic tissue was the same as in the original specimen (**Figure 3**).
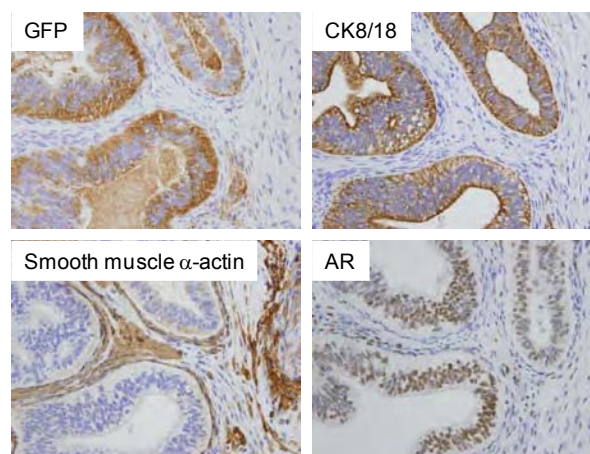
Figure 3: Second round tissue recombination with rat UGM and hESCs induced differentiation of endoderm structures. Markers included GFP to track ENVY hESCs, cytokeratins 8/18 for epithelial cells, smooth muscle α-actin for stromal cells and androgen receptor (AR) for prostate-like cells.

Predicted Outcome: We predicted that we could optimize the conversion of prostatic glands from human embryonic stem cells providing an unlimited supply of human prostatic normal tissue from which to isolate epithelial stem cells in further tasks.

Actual Outcome: Using the strategy outlined in the research plan, we failed to show 100% conversion prostatic glands from hESCs as we predicted. In fact, the amendments we made to our original protocol made little or no improvement over our initiation findings reported in 2006. We used both approaches described, including the alternative approach of a two-step recombination approach. This outcome forced us to consider other alternative strategies to test our hypothesis of prostate cancer initiation by stromal cells. When conducting studies for the following tasks, we included a benign prostatic epithelial cell line, BPH-1 cells, that has been used extensively in tumor stroma recombination experiments in the past (*6, 7*).

Task 2 (Aim 1.2): *To isolate normal human prostatic epithelial cells, including putative stem cells, from hESC-derived normal human prostate* [Years 0.5-2.5].

a. Generate tissue recombinants from protocol optimized in task 1, and harvest tissues from host SCID mice.

This was completed as described above. We also included another epithelial cell source to isolate putative stem cells, since the prostatic tissue available from Aim 1 was a mixed population. We included the BPH-1 cell line, which we is derived from human BPH tissue, and immortalized with SV40 T antigen (*8*). This cell line contains a population of CD133+ cells and has been shown to reliably differentiate into prostatic ducts under the influence of UGM.

b. Separate epithelial cells from stromal cells using collagenase and trypsin from tissue recombinants and isolate epithelial cell populations based on $\alpha2\beta1$ integrin, CD133, CD44 and CD57 cell surface markers. Culture and expand stem cell population if required.

The cell separation was completed successfully for the hESC-derived grafts. It was not necessary for the BPH-1 cell line which is grown *in vitro*. Using both epithelial cell sources, we conducted cell isolation experiments using the cell surface markers listed above. We used MACs beads (Milenyi Biotech) as per manufactures instructions. We were able to isolate sufficient cells for tissue recombination experiments, without the need to culture the cells further. The proportion of cell populations isolated is tabled below:

Table 1: % of CD133 cells.

| Cell Type	Stem cells ($\alpha2\beta1\text{int}^{hi}$/CD133+/CD44+)	TA cells ($\alpha2\beta1 \text{ int}^{hi}$/CD133-/CD44+)	Secretory cells ($\alpha2\beta1 \text{ int}^{lo}$/CD57+)
hESC-derived epithelium	0.19%	23.75%	76.06%
BPH-1 cells	0.05%	28.84%	71.11%

c. Generate tissue recombinants of epithelial cell populations including stem cells (CD44+/CD133+), transiently amplifying basal cells (CD44+/CD133-) and committed secretory cells (CD44-/CD57+) together with rodent UGM

or SVM using collagen gel technique and sub-renal grafting into male SCID mice.

We have completed a series of experiments using all three cell populations described above, from both hESC-derived epithelial (either undifferentiated or pre-differentiated; the results from either cell source were comparable) and BPH-1 cells. There were three experimental groups analyzed for each cell line:

1. rUGM + Stem cells from hESC-derived epithelia or BPH-1 cells
2. rUGM + Transient amplifying cells from hESC-derived epithelia or BPH-1 cells
3. rUGM + Secretory cells from hESC-derived epithelia or BPH-1 cells

   d. Harvesting and analysis of tissue recombinants including immunohistochemistry for morphological analysis and cell death/proliferation markers.

Analysis of grafts from the above experimental groups was conducted using immunohistochemistry for morphological analysis. The hESC-derived epithelia and human BPH-1 cells survived in all tissue recombinants, regardless of the sub-population of cells used (**Figure 4**). There was significantly less growth from the secretory cells (from both epithelial sources), but the amount of tissue generated from stem or transient amplifying cells was comparable. We present data from the latter two experimental groups:
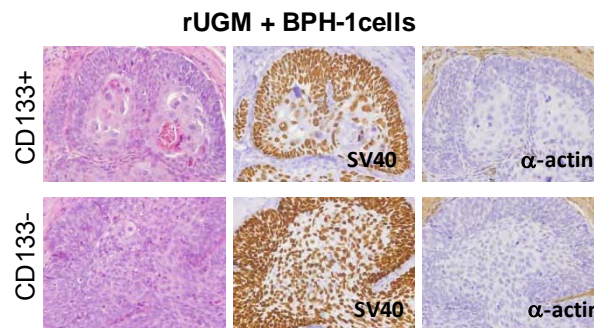
Figure 4: Induction of prostatic ducts in BPH-1 cells. Tissue recombinants of rat UGM plus BPH-1 cells that were enriched as CD133+ and CD133- both formed prostatic ducts, as seen in H&E micrographs, SV40 immunostaining in BPH-1 cells and smooth muscle α-actin in surrounding stroma.

Predicted Outcome: We set out to isolate and characterize epithelial cell populations from hESC-derived prostatic tissue recombinants including stem cells, transiently amplifying basal cells and terminally-differentiated secretory epithelial cells by selecting the epithelial cell population with the greatest regenerative potential for further studies in tasks 3 and 4 (most likely CD44+/CD133+ cells).

Actual Outcome: For these studies, we used hESC-derived epithelia as well as an additional epithelial cell source, BPH-1 cells. This was based on the limiting results produced in task 1, that failed to optimize the efficiency of prostatic differentiation in our hESC cell differentiation system. Since there were epithelial cells obtained from multiple other structures (either endoderm-derived for pre-differentiated cells, or derived from all three germ layers using

undifferentiated hESCs), the am ount of pr ostatic differentiation observed from isolated cell populations was not expected to be high. Therefore, we included an alternative human benign prostatic epithelial cell line to test the d ifferentiation capacity of t he individual cell sub-populations.

Our results showed that as expected, prostatic differentiation from hESC-derived epithelia was poor, regardless of the sub-population of cells used, providing no information about the differentiation capacity of the putati ve stem cells in this model system. Using BPH-1 cells, we reproducibility showed extensive growth and di fferentiation from both the s tem (CD133+) and transient amplifying (CD133-) cell populations. This was the fi rst demonstration of s uch extensive differentiation from the CD133- cell population *in vivo*; *in vitro* studies showed limited differentiation capacity from primary human cells (*9*). Providing the cells with a supportive *in vivo* environment had a major influence on thei r survival and gr owth, indicating that regenerative capacity of prostatic epithelia is not restricted to the CD133+ stem cells as originally predicted. This data also supports the fi ndings of V ander Griend and c o-workers who describe differentiation potential of CD133- prostatic epithelia (*10*).

Task 3 (Aim 2.1): *To determine whether CAFs can initiate tumorigenesis in normal human prostatic epithelia* [Years 2-2.5]*.

    a.   Patient recruitment and tissue collection from men with prostate cancer at radical prostatectomy, for collection of c arcinoma-associated prostatic fibroblasts (CAFs) and normal prostatic fibroblasts (NPFs).

We generated up to 9 primary CAF lines during the funded per iod (and patient-matched NPFs from non-malignant adjacent radical prostatectomy tissue). These cell lines were established and frozen into stocks at passage 1. We thawed these cells for use up to pas sage 3, at w hich time their tumorigenic capacity was reduced. The patient cohort we selected for the generation of CAFs was Gleason 7 (3+4 or 4+3) to reduce the variability of cell lines. This is a valuable resource to our laboratory, as well as the wider research community.

    b.   Establish culture of CAFs and NPFs and determine growth characteristics and properties of CAFs compared to NPFs using immunohistochemistry.

We routinely conduct comparative analysis on the CAF and NPF lines during establishment and culture past passage 1. B oth CAFs and N PFs grow at a s imilar rate, and s how similar expression of key prostatic markers, including vimentin, smooth muscle and androgen receptor, whilst being immuno-negative for epithelial markers such as cytokeratins.
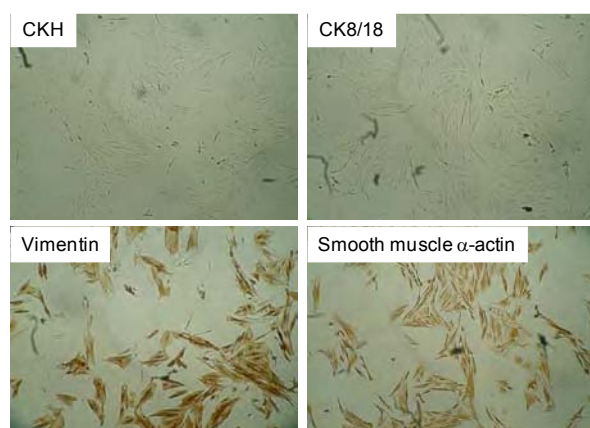
Figure 5: Epithelial and stromal markers in CAF cultures. Immunohistochemistry for high molecular weight cytokeratins (CKH) and cytokeratins 8/18 were negative (indicating lack of epithelial cells, whilst vimentin and s mooth muscle α-actin were positive in stromal cells.

c. Generate tissue recombinants from protocol optimized in task 1, harvest tissues from host SCID mice and isolate epithelial cell populations as optimized in task 2.

Based on the results in Task 2, we restricted our studies to the BPH-1 cell line, since hESC-derived epithelia produced few prostatic ducts for analysis. In an attempt to address the aim of testing which prostatic epithelial population is tumorigenic, we used BPH-1 cells that routinely produce differentiated prostatic ducts. As a positive control, we know that unsorted BPH-1 cells form malignant tumors under the influence of CAFs (but not NPFs) (*6*) (and our unpublished data).

d. Generation of tissue recombinants of CAFs or NPFs together with prostatic stem cells (most likely CD44+/CD133+ cells) isolated and characterized in task 2 using collagen gel technique and sub-renal grafting into male SCID mice. CAFs will also be recombined with BPH-1 cells as positive controls.

We have completed a series of experiments using BPH-1 cells as either unsorted, stem cells (CD133+) or transient amplifying cells (CD133-). All cell types were recombined with CAFs and NPFs.

1. CAF or NPF + unsorted BPH-1 cells
2. CAF or NPF + CD133+ BPH-1 cells
3. CAF or NPF + CD133- BPH-1 cells

e. Harvesting and analysis of tissue recombinants including immunohistochemistry for morphological analysis and cell death/proliferation markers.

Analysis of grafts from the above experimental groups was conducted using immunohistochemistry for morphological analysis. Whereas CAFs induced malignant transformation of unsorted BPH-1 cells (*6*) (defined as invasive carcinoma), sorted BPH-1 cell showed differences, such that CD133+ formed intact cords, but CD133- cells gave rise to invasive tumors (**Figure 6**). We present the data below:
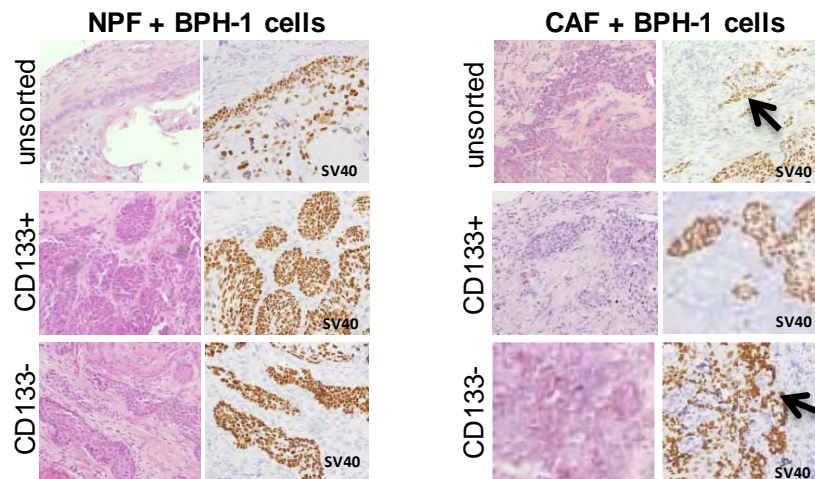
Figure 6: Tissue recombinants of CAF or NPH with BPH-1 cells. BPH-1 cells were grafted as unsorted fractions, or enriched for CD133+ and CD133-. CAFs induced invasive tumors (arrow) in unsorted and CD133- BPH-1 cells, but not CD133+ BPH-1 cells. NPF grafts were always non-malignant.

Predicted Outcome: We predicted that C AFs would either initiate tumorigenesis or not; regardless, the outcome of this task will be a significant contribution to the cancer biology field. If our hypothesis was proven, we could provide definitive evidence that human prostate cancer can be initiated by tumor stroma; therefore the stroma becomes a novel target for chemoprevention. Alternatively, we will reveal that C AFs could only cause tumorigenesis in epithelial cells that have previously acquired genetic alterations.

Actual Outcome: Our results were unexpected, but as predicted, make a s ignificant new contribution to our knowledge of prostate cancer cell biology. We showed that CD133- transient amplifying cells are more susceptible to tumorigenesis by prostatic tumor stroma, whereas putative CD133+ stem cells were protected, and failed to show malignant differentiation. This data is in agreement with data presented by Vander Griend that CD133+ cells may not be the tumor cell of origin in prostate cancer (*10*).

Task 4 (Aim 2.2):   *To determine that CAFs can only promote progression in genetically modified human prostatic epithelia* [Years 2-3].

During the funded period, there was a seminal publication in Nature demonstrating that loss of stromal PTEN can induce epithelial tumorigenesis in mouse mammary models (*11*). This data was exciting to the prostate field, since it is also possible that PTEN plays a critical role in prostatic tumor stroma, during either initiation of progression of prostate cancer. We will consider examining PTEN expression in our CAFs and NPFs from human patients, and determine whether this tumor suppressor is active in prostatic tumor stroma, as well as epithelial cells.

> a.   Continue patient recruitment and ti ssue collection from prostate cancer patients for CAF and NPF collection and characterization.

As described for Aim 3, we generated a significant resource of up to 9 primary CAF lines during the funded period. These were fully characterized and validated in recombination assays with BPH-1 cells to prove they were tumourigenic.

> b.   Generation of knockdown hESCs using lentiviral shRNA constructs for key prostate cancer genes *RB* and *PTEN*.

We were unable to perform these studies, since our initial findings in Aims 1 and 2 showed that the hESC-induced model was not sufficient to derive normal prostatic stem cells, and we used BPH-1 cells as an alternative approach.

BPH-1 cells are an epithelial cell line derived from human prostate tissue obtained by transurethral resection (*8*). Primary epithelial cell cultures were immortalized with SV40 large T antigen and are non-tumorigenic in nude mice following subcutaneous injection or subrenal capsule grafting. They express the SV40 large T antigen, increased levels of p53, and cytogenetic analysis by G-banding demonstrated an aneuploid karyotype with a modal chromosome number of 76 (range 71 to 79, n = 28) and 6 to 8 marker chromosomes. This abnormal phenotype means that these cells are already susceptible to carcinogenesis, especially by CAFs in tissue recombination, and therefore the proposed knockdown studies for *RB* or *PTEN* were not appropriate.

Instead, we performed some genetic analysis of the PTEN signaling pathway in human CAF stromal cells, based on the study in mouse mammary stroma by Trimboli and colleagues (*11*). In order to determine whether PTEN signalling was active in CAFs compared to NPFs, we performed a Human 1.0st genome wide Affymetrix array on quadruplicate samples of one patient matched CAF/NPF line (**Figure 7**). Although PTEN gene expression was not altered, we saw significant increase in AKT3 activity, an indicator of PI3K pathway activation, potentially due to loss of PTEN in CAFs. The association between PTEN signalling and tumorigenic potential warrants further investigation.
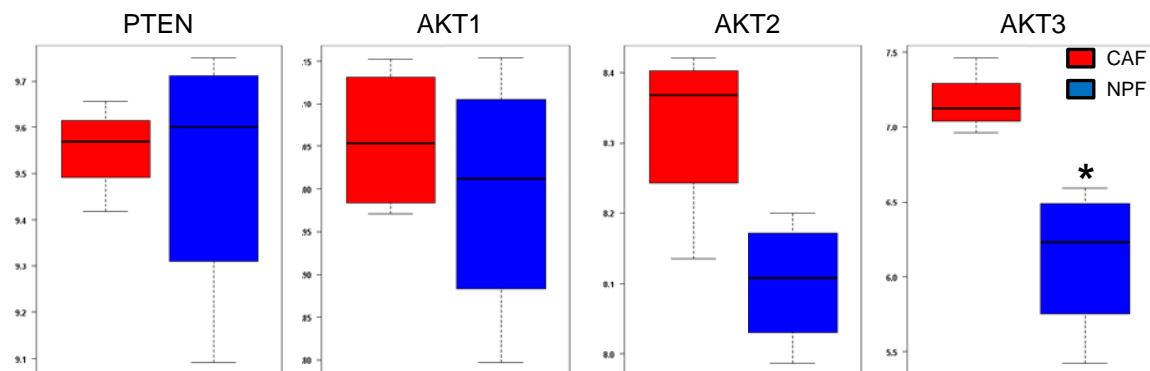
Figure 7. Expression of PTEN or PI3K pathway genes in CAFs (red) or NPFs (blue). Data are represented as boxplots. Numbers are fold difference between CAF and NPF (n=4 replicates, p<0.05).

c. Characterize knockdown *RB* and *PTEN* hESCs in terms of gene and protein expression and growth characteristics *in vitro*.

Experiments were not possible due to inefficiency of hESC-derived prostatic epithelia.

d. Generate tissue recombinants from protocol optimized in task 1 using *RB* or *PTEN* knockdown hESCs, harvest tissues from host SCID mice and isolate epithelial cell populations as optimized in task 2.

Experiments were not possible due to inefficiency of hESC-derived prostatic epithelia.

e. Generation of tissue recombinants of CAFs or NPFs together with stem cells from genetically altered hESCs (knockdown of *RB* or *PTEN*) using collagen gel technique and sub-renal grafting into male SCID mice. CAFs will also be recombined with BPH-1 cells as positive controls.

Experiments were not possible due to inefficiency of hESC-derived prostatic epithelia.

f. Harvesting and analysis of tissue recombinants including immunohistochemistry for morphological analysis and cell death/proliferation markers.

Predicted Outcome: We predicted that a single genetic defect (loss of *RB* or *PTEN*) would increase susceptibility of prostate stem cells to malignant transformation by CAFs, providing unequivocal evidence that CAFs can only initiate tumorigenesis and that the primary genetic insult must occur in the epithelium for carcinogenesis to be initiated. In addition, successful initiation of prostate cancer would result in the production of novel malignant tumor models in which both the cancer cells and surrounding microenvironment are of human origin and grown *in vivo* where cell-cell interactions and hormonal milieu are conserved.

Actual Outcome: Unfortunately we were unable to carry out these studies as planned, since our initial findings that hESC-derived grafts were insufficient in producing sufficiently pure prostatic epithelia. The BPH-1 cells we used as an alternative approach to study tumor initiation in stem cells, were already initiated with genetic defects, including *RB* and *PTEN* mutations, and therefore further mutations using these cells was inappropriate.

Instead, we focused our studies on PTEN signaling in the CAFs, which are proven to be tumorigenic. In these stromal cells, it is possible that loss of PTEN leads to activation of PI3K signaling, which contributes to its tumor potential. Dissecting the genetic changes in CAFs will significantly advance our ability to treat prostate cancer, since the data generated in this project confirm that the tumor stroma is a significant therapeutic target.

Key Research Accomplishments

- Generated significant resource of human stromal cells including CAF derived from prostate cancer specimens and NPFs from adjacent non-malignant tissues.

- Generation of hESC-derived epithelial cells (although the proportion of prostatic tissue is low).

- Generated new data that demonstrate that CD133+ and CD133- cells show equal differentiation potential in tissue recombination with embryonic prostatic stroma.

- Generated new data that CD133+ cells are resistant to tumorigenic influence by prostate cancer tumor stroma.

- Generated new data to show that PTEN and PI3K signaling is a potential target in prostatic tumour stroma.

Reportable Outcomes

Manuscripts:

1. Risbridger GP & Taylor RA (2011) The complexities of identifying a cell of origin for human prostate cancer. *Asian Journal of Andrology*. 13(1):118-9.

   - Invited commentary in response to a publication in Science by *Goldstein et al* reporting identification of cancer cells-of-origin from human prostate cancer.

2. Taylor RA, Toivanen R, Risbridger GP (2010) Stem Cells in Prostate Cancer – Treating the Root of the Problem. *Endocrine-Related Cancer* 17(4):R273-85. *(IF 5.683)*

   - Evidence of significant impact of this article include 1) the citation of this work by Ken Garber in his article in Journal of National Cancer Institute titled "**Tale of two cells: Discovering prostate cancer cells of origin**", including figure from our review reproduced with permission and 2) citation on a poster by Nature Reviews Cancer on Cancer Stem Cells. *Significant publication included in appendix.*

3. Yao M, Taylor RA, Richards MG, Sved P, Wong J, Eisinger D, Xie C, Salomon R, Risbridger GP, Dong Q (2010) Prostate regenerating capacity of cultured human adult prostate epithelial cells. *Cells Tissues Organs* 191(3):203 - 212 *(IF 2.6)*

4. **Risbridger GP**, Davis ID, Birrell S, Tilley W (2010) Breast and prostate cancer: more similar than different. **Nature Reviews Cancer** 10(3):205-12 *(IF 30.8)*

5. Taylor RA, Risbridger GP (2008) The path towards identifying prostatic stem cells. **Differentiation** 76(6):671-681 *(IF 3.745)*

6. Taylor RA, Risbridger GP (2008) Prostatic tumour stroma: a key player in cancer progression. **Current Cancer Drug Targets** 8(6):490-7 *(IF 5.677)*

7. Risbridger GP, Taylor RA (2008) Regulation of prostatic stem cell niche in health and disease. **Endocrinology** 149(9):4303-4306 *(IF 5.236)*

Abstract presentations:

1. Taylor RA, Toivanen R, Wang H, Frydenberg M, Pedersen J, Collins AT, Maitland N, Risbridger GP (2011) CD133- intermediate/transient amplifying cells undergo tumour-initiation by prostatic tumour stroma and hormonal carcinogenesis. The Innovative Minds in Prostate Cancer Today (IMPacT) Conference, Orlando, Florida, USA. (Poster presentation)

2. Risbridger GP, Hussein S, Toivanen R, Wilkinson S, Frydenberg M, Pedersen J, Ellem SJ, Taylor RA (2011) Cancer stem cells and tumour stroma. Lorne Cancer Conference, Victoria (Poster presentation)

3. Taylor RA, Buchanan G, Toivanen R, Trotta A, Frydenberg M, Pedersen J, (2010) Loss of androgen receptor activity in prostatic carcinoma-associated fibroblasts contributes to malignant transformation of intermediate/transit amplifying (CD133-) cells. *Lorne Cancer Conference*, Victoria. (Poster presentation)

4. **Taylor RA**, Toivanen R, Frydenberg M, Pedersen J, Collins AT, Maitland N, Risbridger GP (2009) Prostatic tumour stroma induces tumourigenicity of intermediate/transit amplifying (CD133-) cells. *The Inaugural Ludwig Institute for Cancer Research Translational Oncology Conference,* Melbourne (Poster presentation; ***Awarded Poster Prize)**

5. Taylor RA, Toivanen R, Frydenberg M, Pedersen J, Collins AT, Maitland N, **Risbridger GP** (2009) Prostatic tumor stroma induces tumorigenicity of intermediate/transit amplifying (CD133-) cells. *Gordon Research Conference on Stem Cells and Cancer,* Les Diablerets, Switzerland (Poster presentation; ***Attendance via invitation only**)

6. **Taylor RA,** Toivanen R, Hill T, Wang H, Richards M, Pedersen J, Collins A, Maitland NJ, Risbridger GP (2009) Prostatic stroma determines epithelial cell fate of BPH-1 cells, but not PC3 cells, regardless of CD133 status. *The 89th Annual Meeting of the Endocrine Society,* Washington DC, USA. (**[#]This abstract was selected for oral presentation)**

7. Taylor RA, Toivanen R, Pedersen J, Collins A, Maitland NJ, Risbridger GP (2008) Altered differentiation of CD133+ prostatic stem cells by carcinoma-associated fibroblasts. *The Role of Cancer Stem Cells in the Initiation and Propagation of Tumorigenesis; Special conference of American Association for Cancer Research*, Los Angeles, USA (*poster presentation*).

Symposia / Seminar presentations:

International:

1. Risbridger GP (2010) **14th International Congress of Hormonal Steroids and Hormones & Cancer**, Edinburgh, UK (Invited Speaker)

2. Risbridger GP (2009) **9th International Congress of Andrology,** Barcelona, Spain (Invited Speaker) – "*From Human Stem Cells to Prostate Tissues"*

National (Australia):

1. Taylor RA (2010) Prostate cancer tumour stroma. **Annual Meeting of the Prostate Cancer Foundation of Australia**, Gold Coast, Qld, Australia. Invited Speaker

2. Risbridger GP (2009) **Southern Health Research Week**, Monash Medical Centre, Melbourne – *"Prostatic stem cells in health and disease"*

3. Ribsridger GP (2009) **10th National Prostate Cancer Research Forum,** Melbourne – *"Stromal-epithelial interactions in prostate disease"*

4. Taylor RA (2009) Invited institute seminar presentation, **Division of Molecular Medicine, Walter and Eliza Hall Institute (WEHI),** Melbourne – *"Stromal-epithelial (stem cell) interactions in prostate development and cancer"*

5. Taylor RA (2009) Invited institute seminar presentation, **Department of Anatomy, Monash University,** Melbourne – "*Stromal microenvironment and stem cell interactions in the prostate gland* "

Generation of resources:

- **Development of primary stromal cell lines from human prostate cancer tissues.** This award has supported the establishment of carcinoma-associated fibroblasts and normal prostatic fibroblasts from up to 15 patients. Specimens are collected at the time of radical prostatectomy and primary cell lines have been characterized and validated for their tumourigenic potential. The generation of this resource has led to this research team obtaining further grant funding for related projects.

Other funding arising from this award:

1. **Prostate Cancer Foundation of Australia Project Grant** (2011 – 2012) "*Imbalance of Stromal Steroid Receptor Signalling Contributes to Prostate Cancer Progression" $250,000AUD* [PI: Taylor RA, Buchanan G; ID PG 0810] [based on development of primary stromal cell line resource supported by this award]

2. **National Health and Medical Research Council Project Grant** (2010-2012) "*Defining Stromal – Cancer Cell Interactions for Xenografting Human Prostate Cancer" $583,000*AUD [PIs: Risbridger GP, Taylor RA, Berman DM; ID 606492] [based on expertise in stromal-epithelial signalling supported by this award]

Research opportunities arising from this award:

1. Dr. Renea Taylor was selected for the **Monash Research Accelerator Program** in 2010.   This is a new initiative by Monash University that aims to recognise, reward and accelerate the career development of the highest performing early to mid career researchers (offered to top 30 performing research or teaching staff).

List of personnel receiving pay from this research effort:

1. Ms. Hong Wang (Research assistant)
2. Dr. Brindi Niranjan (Research assistant)
3. Ms. Roxanne Toivanen (Graduate student)

Conclusion

In summary, these data failed to support the hypothesis that prostatic tumour stroma is an initiator of tumorigenesis, but rather our findings indicated that preceding events in the epithelial cells are most likely required to begin the process of malignancy. We went on to prove that CAF-induced malignancy was restricted to intermediate/transient amplifying (CD133-) cells, but not stem (CD133+) cells under the influence of stroma. This is an important finding, and provides new information regarding the initiation of prostate cancer, and the potential cancer cell of origin.

During the funded period, there were several reports that identified normal prostatic stem cells, that can be cancer cells of origin, from both human and mouse tissues (*12-14*). These rare cells have been identified in both the basal and luminal cell compartments, and it is now feasible to use these newer methods to isolate stem cells to test their tumor potential by stroma.

Defining the role of prostatic tumour stroma in the initiation of carcinogenesis significantly impacts on the field of prostate cancer (and other major cancers). These findings, based on an innovative approach using human prostate stromal cells and embryonic stem cells, have provided fundamental advances to our understanding how cancer is initiated and thus may be prevented or treated. Our current data implicate both transient amplifying epithelial cells and stromal components of the tumor as therapeutic targets.

References

1. R. A. Taylor, G. P. Risbridger, *Curr Cancer Drug Targets* **8**, 490 (Sep, 2008).
2. G. R. Cunha *et al.*, *J. Androl.* **13**, 465 (1992).
3. R. A. Taylor *et al.*, *Nat Methods* **3**, 179 (Mar, 2006).
4. K. A. D'Amour *et al.*, *Nat Biotechnol* **23**, 1534 (Dec, 2005).
5. K. A. D'Amour *et al.*, *Nat Biotechnol* **24**, 1392 (Nov, 2006).
6. A. F. Olumi *et al.*, *Cancer Res* **59**, 5002 (Oct 1, 1999).
7. M. Ao *et al.*, *Cancer Res* **67**, 4244 (May 1, 2007).
8. S. W. Hayward *et al.*, *In Vitro Cell Dev Biol Anim* **31**, 14 (Jan, 1995).
9. G. D. Richardson *et al.*, *J Cell Sci* **117**, 3539 (Jul 15, 2004).
10. D. J. Vander Griend *et al.*, *Cancer Res* **68**, 9703 (Dec 1, 2008).
11. A. J. Trimboli *et al.*, *Nature* **461**, 1084 (Oct 22, 2009).
12. A. S. Goldstein, J. Huang, C. Guo, I. P. Garraway, O. N. Witte, *Science* **329**, 568 (Jul 30, 2010).
13. X. Wang *et al.*, *Nature* **461**, 495 (Sep 24, 2009).
14. K. G. Leong, B. E. Wang, L. Johnson, W. Q. Gao, *Nature* **456**, 804 (Oct 22, 2008).

Appendices

Stem cells in prostate cancer: treating the root of the problem

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Abstract

Prostate cancer is a hormone-dependent, epithelial-derived tumor, resulting from uncontrolled growth of genetically unstable transformed cells. Stem cells are therapeutic targets for prostate cancer, but as disease progression occurs over decades, the imperative is to identify and target the cancer-repopulating cell (CRC) that maintains malignant clones. In order to achieve this goal, we will review the current knowledge of three specific types of cells, their origins, and their differentiation potential. The first is the normal stem cell, the second is the cancer cell of origin, and the third is the CRC. Specifically, we review three proposed models of stem cell differentiation in normal tissues, including linear, bidirectional, and independent lineages. We consider evidence of the cancer cell of origin arising from both basal and luminal cells. Finally, we discuss the limited data available on the identity and characterization of CRCs in localized and castrate-resistant prostate cancer, which is where we believe the focus of future research efforts should be directed. Ultimately, understanding the intrinsic or extrinsic influences that dictate the behavior of these unique cells will be instrumental in facilitating the development of new therapeutic targets for prostate cancer.

Endocrine-Related Cancer (2010) **17** R273–R285

Introduction

Prostate cancer is a major cause of morbidity and mortality in men around the world, being the most common solid tumor. In America, ∼6 out of every 50 men over the age of 50 will be diagnosed with prostate cancer in his lifetime. In 2006 alone, the American Cancer Society reported 234 460 men diagnosed and 27 350 deaths from prostate cancer (Penson & Chan 2007). These statistics underscore the significance of this cancer and predict the significant health burden on our aging population. Prostate cancer is treated by surgery or radiation when confined to the organ at diagnosis, and as it is an androgen-dependent malignancy, androgen deprivation therapy (ADT) is used to control the disease, if disease relapse occurs. However, cancer cells can adapt to androgen-depleted conditions and patients inevitably progress from hormone sensitive to develop castrate-resistant prostate cancer (CRPC). Carcinogenesis occurs in the prostatic epithelium, and results in sequential disruption of coordinated reciprocal signaling between stroma and epithelium (Hayward *et al*. 1997).

In this review, we consider stem cells as cellular targets for prostate cancer therapies. To avoid confusion throughout our review, we propose to discuss prostatic stem cells during disease progression focusing on three specific types of cells, their origins, and their differentiation potential. The first is the normal stem cell, the second is the cancer cell of origin, and the third is the cancer-repopulating cell (CRC). Conventionally, stem cells are defined by their ability to self-renew and differentiate into progeny. In normal tissues, stem cells are the epithelial populations with full lineage potential that are proven to regenerate tissue-specific progeny (Potten & Loeffler 1990, Watt & Hogan 2000). We also consider the cancer cell of origin. This is not necessarily a stem cell in normal tissue, but may also be a progenitor, which is susceptible to malignant transformation. Thirdly, we will discuss the CRC, defined as a population of biologically distinct tumor cells possessing stem cell properties. These cells have the ability to self-renew, repopulate the tumor after chemotherapy, and play a role in subsequent metastasis (Bonnet & Dick 1997,

Reya *et al*. 2001, Wicha *et al*. 2006). Other terms which have been used for CRCs are 'cancer stem cells' or 'cancer-initiating cells', but this terminology does not distinguish adequately between a cancer cell of origin and a CRC. Thus, for the purposes of this review, we will avoid these terms and instead use stem cell, cancer cell of origin, and CRC, as defined earlier.

Of all these stem cell types, the most appropriate therapeutic target is the CRC. However, there is a need to know the relationship between stem cells in normal tissue, cancer cell(s) of origin, and CRCs. Herein, we present three proposed models of stem cell differentiation in normal tissues, including linear, bidirectional, and independent lineages. We present evidence for cancer cell(s) of origin from progenitors of both basal and luminal cells. Finally, we discuss the limited data available on the identity and characterization of CRCs in localized and advanced prostate cancer, which we believe is where future research efforts should be directed.

Prostatic stem cells in normal tissue

Classification of prostatic epithelial cell types in normal tissue

In order to discuss the role of stem cells in normal tissues, it is important to identify the key cell types of the normal epithelium. Prostatic epithelium is composed of multiple differentiated cell types, including basal, luminal (secretory), and neuroendocrine cells. In addition, an intermediate cell type that shares properties of both luminal and basal cells is described (De Marzo *et al*. 1998, Wang *et al*. 2001, Uzgare *et al*. 2004, Signoretti & Loda 2007). Luminal secretory cells make up the majority of the epithelial layer and because they express androgen receptors (ARs), they can respond directly to androgens by simulating production and secretion of prostatic proteins, such as prostate-specific antigen (PSA) and prostatic acid phosphatase (Coffey 1992, Hudson 2004, Kurita *et al*. 2004). The basal cells exist as one or two layers attached to the basement membrane below the luminal cells (McNeal 1981, 1988, Kurita *et al*. 2004, Heer *et al*. 2007) and can be distinguished from other prostatic cells by their morphology, ranging from small, flattened cells with condensed chromatin and small amounts of cytoplasm to cuboidal-like cells with an increased cytoplasm and more open-appearing chromatin. In the human prostate, basal cells form a continuous layer, whereas in other species they are more scattered in appearance. This is reflected in the ratio of basal:luminal cells, which is ∼1:1 in human prostate, whereas the average ratio in other species, such as mouse, dog, monkey, and rat, is ∼1:7 (El-Alfy *et al*. 2000). Basal cells usually have low AR expression and exclusively express *p63* (a homolog of the tumor suppressor gene *p53*; Signoretti *et al*. 2000, Signoretti & Loda 2007). Neuroendocrine cells are the least studied epithelial cell population and are believed to regulate prostate growth and development through endocrine–paracrine actions (Bostwick & Dundore 1997). They are rare cells located in the luminal layer of the epithelium, together with the secretory cells they tend to be more abundant in the major ducts and more sparsely present in acinar tissue (Abrahamsson 1999).

Prostatic epithelial cells are identified by their morphological appearance, location, and also distinct patterns of marker expression. Basal cells express cytokeratins (CKs) 5 and CK14, but not CK8 or CK18. Luminal cells are devoid of basal cell markers, expressing CK8 and CK18, but not CK5 or CK14. Intermediate cells express CKs of both basal and luminal cells (CKs 5, 14, 8, and 18; De Marzo *et al*. 1998, Wang *et al*. 2001, Uzgare *et al*. 2004, Signoretti & Loda 2007; see Fig. 1). Throughout this review, these cell types will be denoted as basal ($CK5^+8^-$), intermediate ($CK5^+8^+$), and luminal ($CK5^-8^+$) cells.

Identity and characterization of prostatic stem cells in normal tissue

Although the prostate is a slow growing organ with limited cycles of cell proliferation and apoptosis, prostatic stem cells exist within the epithelium, which are capable of regenerating the adult organ (DeKlerk & Coffey 1978). Although stem cells in the normal prostate are not a direct target for cancer therapies, fundamental understanding of their identity and characteristics provide an imperative basis to our understanding of cancer cell(s) of origin and CRCs. There is a significant controversy in the field with regard to these cells based on conflicting data, leading to multiple proposed differentiation hierarchies. We review three generally accepted models of stem cell differentiation in normal tissue, including linear, bidirectional, and independent lineages. The true stem cell hierarchy(s) is likely to involve a combination of all models, but more data are required to resolve this issue.

Linear differentiation model

Adult prostatic stem cells were originally postulated to reside within the basal cell compartment because of the ability of the prostatic epithelium to regress and regenerate from residual basal cells after repeated
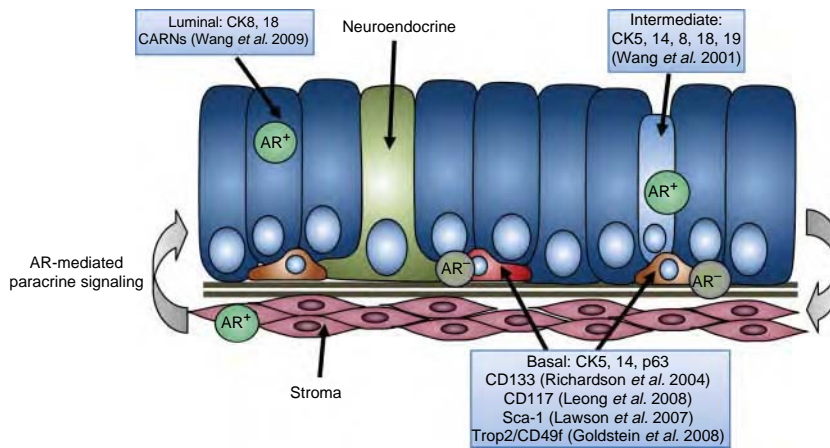
Figure 1 Cellular identity of prostate stem cells. The prostate in composed of stromal and epithelial compartments that communicate through reciprocal communication. The epithelium consists of several identifiable cell types, including basal, intermediate, luminal, and neuroendocrine cells that have defining cytokeratin (CK) profiles and differing androgen receptor (AR) expression. Putative stem cells are identified in the AR$^-$ basal compartment, based on sorting for cell surface markers including CD133, CD117, Sca-1, Trop2, and CD49f, or in the luminal compartment where AR$^+$ castrate-resistant *Nkx3-1*-expressing cells (CARN) reside.

cycles of castration and testosterone replacement (DeKlerk & Coffey 1978, Kyprianou & Isaacs 1988, Montpetit *et al*. 1988, Verhagen *et al*. 1988). Biologically, basal cells exhibit many stem cell characteristics, including their relatively undifferentiated state, high proliferative capacity, protection from apoptosis, and a long life span (Potten & Loeffler 1990, Bonkhoff *et al*. 1994, De Marzo *et al*. 1998, Foster *et al*. 2002). A linear hierarchical model of stem cell differentiation in prostatic epithelia is defined by Isaacs & Coffey (1989), where stem cells within the basal layer give rise to one stem cell copy (self-renewal) and one multipotent progenitor cell (or transient amplifying cell), by asymmetric cell division. During expansion, progenitor cells translocate toward the luminal cell layer and gain either exocrine or neuroendocrine characteristics through an intermediate cell phenotype (Bonkhoff 1996, De Marzo *et al*. 1998, van Leenders & Schalken 2001; Fig. 2A). This linear model is similar to other regenerative tissues such as bone marrow, skin, intestinal tract, and squamous epithelium, as demonstrated by studies that use cell surface markers to isolate enriched populations that are identified as putative stem cells based on their regenerative functionality using *in vitro* and *in vivo* assays.

The ability to isolate and study stem cells in human prostate tissues based on cell surface markers is limited by the availability of healthy tissue from an undiseased human prostate gland. Therefore, most findings are established and extrapolated from the use of mouse models. Several cell surface markers are reported to identify prostate stem cells in the basal cell compartment, including stem cell antigen-1 (Sca-1, also known

as Ly6a), ALDH, CD133 (Prom1), Trop-2, and CD44 (Liu *et al*. 1997, Burger *et al*. 2005, Xin *et al*. 2005, Lawson *et al*. 2007, Tsujimura *et al*. 2007, Goldstein *et al*. 2008, Yao *et al*. 2010). However, many nonstem cells in the mouse prostate also express these markers. Most recently, Leong *et al*. (2008) identified CD117 (c-kit, stem cell factor receptor) as a new marker of a rare adult mouse prostatic stem cell population that fulfills all the functional characteristics of stem cells including self-renewal and full differentiation potential. Used in combination with other stem cell markers, single cells defined by the phenotype Lin$^-$Sca-1$^+$CD133$^+$CD44$^+$CD117$^+$ regenerate prostatic epithelium that consists of all epithelial cell types and produces secretions *in vivo*. Long-term self-renewal capacity is evident by their ability to regenerate tissue after serial isolation and subsequent transplantation (Leong *et al*. 2008). CD117 expression is predominantly localized to the proximal region of the mouse prostate and is upregulated after castration-induced prostate involution, consistent with prostate stem cell identity and function. CD117$^+$ cells are predominantly basal (CK14$^+$) in the mouse and exclusively basal (*p63$^+$*) in the human (Leong *et al*. 2008). This landmark paper describes single basal cells in the adult mouse prostate with multipotent, self-renewal capacity, defined by CD117 expression.

Although mouse models provide sufficient information relating to stem cells in normal tissues, translation to human tissues is inadequate. The majority of literature in human prostate is based on an enriched population of $\alpha_2\beta_1$ integrin ($\alpha_2\beta_1^{hi}$) CD44$^+$CD133$^+$ cells described by the Maitland
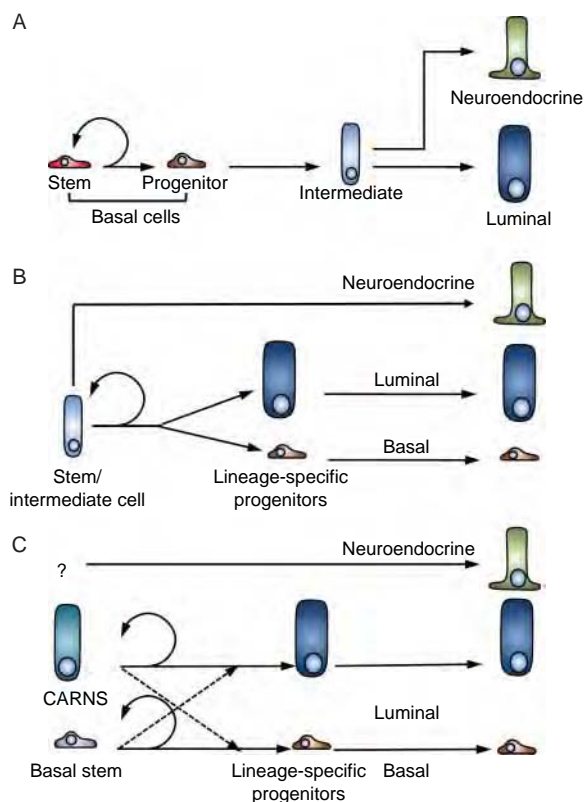
Figure 2 Models of prostate stem cell hierarchies: based on functional and ontological studies, several different models of differentiation hierarchy for putative prostatic stem cells are proposed. (A) Linear hierarchical arrangement: prostatic stem cells are postulated to reside in the basal cell layer, based on functional studies using markers including CD117, Sca-1, Trop2, CD49f, and CD133. In this model, basal cells self-renew, give rise to progenitor (or transit amplifying cells; also basal phenotype), followed by intermediate cells and then terminally differentiated luminal or neuroendocrine cells in a linear manner. (B) Nonlinear hierarchical arrangement: a second differentiation model is proposed where a common stem cell gives rise to a lineage-specific progenitors, which then give rise to distinct cell lineages of basal, luminal, and neuroendocrine cells. This model is most similar to that proven for mammary stem cell differentiation. In the prostate, epithelial cells with an intermediate phenotype (i.e co-expressing markers of both basal and luminal cells) are proposed to house this common stem cell. (C) Independent arrangement: most recently, the identification of luminal stem cells (CARN; based on *Nkx3-1* expression) was demonstrated using expression of *Nkx3-1* in castrate-resistant prostate tissues. This discovery raises the possibility of multiple stem cells within the epithelium that independently gives rise to distinct cell lineages including basal, luminal, and neuroendocrine cells. This model does not exclude the possibility that basal and/or luminal stem cells can be multipotent and generate the opposing lineage as well.

Laboratory (Collins *et al*. 2001, Richardson *et al*. 2004). This subpopulation of cells isolated from benign human prostate tissues show unique ability to form prostatic-like acini over their negative counterparts when xenografted *in vivo* (Richardson *et al*. 2004),

suggesting CD133$^+$ cells represent enriched prostatic stem cells, whereas CD133$^-$ cells are an enriched transient amplifying/progenitor population. Although limited, some studies use other markers in addition to CD133 to identify human prostatic stem cells, including Trop2 and CD49f, which are enriched in the basal epithelium of human prostate and show greater sphere-forming activity *in vitro* (Goldstein *et al*. 2008). Testing of other markers, including CD117, is warranted, as this marker is also expressed in basal cells of benign human prostate (Leong *et al*. 2008), although translation of other cell surface markers from mouse to human, such as Sca-1, is problematic because expression is not shared between species. Therefore, we currently have more functional evidence for prostatic stem cells in murine tissues than in human tissues.

Bidirectional differentiation model

The linear hierarchical model where CK5$^+$8$^-$ basal cells contain the prostatic stem cell population was reviewed by Wang *et al*. (2001), who comprehensively mapped the pattern of CK expression in mouse and human tissues during development and in the mature prostate (Wang *et al*. 2001). They postulated that if prostatic stem cells are located in the urogenital sinus epithelium (UGE), then CK5$^+$8$^-$ basal cells would be enriched in UGE tissues. However, they only detected CK5$^+$8$^+$ intermediate cells (that expressed the full complement of CKs) in human, mouse, and rat fetal UGE (as well as mature tissues), and therefore proposed that CK5$^+$8$^+$ intermediate cells, and not CK5$^+$8$^-$ basal cells, house the prostatic stem cell population that can divergently give rise to basal or luminal cells in a bidirectional manner (Wang *et al*. 2001; Fig. 2B).

A second piece of data that questions the basal cell origin of prostate stem cells, is the generation of prostatic tissue from the *p63*-knockout mouse (Kurita *et al*. 2004). Grafting and rescue studies using this mouse model resulted in mature epithelium (containing all prostatic cell lineages) that undergoes several rounds of serial regression/regeneration in the absence of basal cells (Kurita *et al*. 2004). Again, these data fail to support the suggestion that CK5$^+$8$^-$ basal cells are the 'only' source of prostatic stem cells, and indicate the possible existence of a distinct multipotent stem cell in the intermediate or luminal cell population.

The bidirectional model of prostatic stem cells is similar to the stem cell model of the mouse mammary gland, where the normal stem/progenitor cell hierarchy

is better documented. Mammary stem cells (MaSCs) give rise to a common (bi-potent) progenitor, which generates distinct luminal and myoepithelial progenitors that develop into independent lines of differentiated cell types, including ducal, alveolar, and myoepithelial cells (Visvader 2009). MaSCs are enriched by sorting for $Lin^-CD24^+CD29^-$, and single-cell assays demonstrate their functionality *in vitro* and *in vivo* (Shackleton *et al*. 2006).

Independent lineage model

Both hierarchical models, including linear and bidirectional pathways, postulate that stem cells are confined to a single-cell type, either basal or intermediate cell. However, most recently new data suggest that the prostate gland may also contain stem cells in the luminal compartment, based on the identification of a rare cell that is castrate resistant. Shen's Laboratory used the expression of the *Nkx3-1* homeobox gene to indentify a luminal cell population that displays stem/progenitor properties during prostate regeneration (Wang *et al*. 2009). By using genetic lineage marking, rare luminal cells that express *Nkx3-1* in the absence of testicular androgens (castrate-resistant *Nkx3-1*-expressing cells, CARNs) are shown to be bi-potent and maintain the capacity to self-renew *in vivo*; single-cell transplantation assays show that CARNs can reconstitute prostate ducts in renal grafts (Wang *et al*. 2009). Functional assays of *Nkx3-1* mutant mice in serial prostate regeneration suggest that *Nkx3-1* is required for stem cell maintenance. As these data do not concur with previous reports of basal cells as stem cells, Wang *et al*. proposed that CARNs may be an additional stem cell, such that prostatic stem cells can reside in both basal and luminal compartments, thereby giving rise to their own cell types, rather than being derived from a common stem cell. This model does not exclude the possibility that basal and/or luminal stem cells can be multipotent and generate the opposing lineage as well (Fig. 2C).

Collectively, we present data to implicate prostatic stem cells in basal, intermediate, and luminal cells, and their respective models of differentiation remain speculative. In our opinion, the proposed models may not be mutually exclusive, and all three of the pathways may be active during some stage of development (or disease progression). We also consider that it is possible that cells displaying stem cell properties occur in more than one cell type and that the characteristics that define a stem cell can be switched on or off depending on their response to extrinsic or intrinsic regulatory factors.

Cancer cell of origin

Although we cannot absolutely define stem cells in normal prostatic epithelium, there is an emerging interest in identifying the cancer cell of origin. We have defined the cancer cell of origin as the epithelial cells in normal prostate glands that are susceptible to malignant transformation and therefore capable of initiating tumorigenesis. In general, cancer can arise from normal stem cells that undergo malignant transformation, as these cells exist for the life of the patient, thereby having greater chance of harboring genetic insults leading to tumorigenesis (Reya *et al*. 2001). Alternatively, transient amplifying or progenitor cells can give rise to malignancy, in a process where more rapidly proliferating cells harbor genetic insults leading to tumor formation (Signoretti & Loda 2007). Without a clear definition of stem cells in normal prostate (and considering there may be more than one), it is difficult to determine whether the cancer cell of origin in prostate cancer is a stem cell, multipotent progenitor/transient amplifying cell, or a more differentiated progeny. Nonetheless, evidence exists that the cellular origin can include both basal and luminal (CARN) cells.

Although putative stem/progenitor cells can reside in $CK5^+8^-$ basal cells, a diagnostic feature of human prostate cancer is the loss of basal cells (Humphrey 2007, Grisanzio & Signoretti 2008). Therefore, prostate cancer can potentially arise from oncogenic transformation of $CK5^+8^-$ basal cells resulting in rapid differentiation to a luminal phenotype, or alternatively from stem or multipotent progenitor cells within the $CK5^+8^+$ intermediate or $CK5^-8^+$ luminal populations where stem cells or CARNs are proposed to reside. This is certainly true in the well-characterized mouse mammary epithelium, where aberrant proliferation of the luminal progenitor population, rather than MaSCs, is shown to be the target for transformation in BRAC1-associated tumors (Lim *et al*. 2009). In addition, the multiple subclassification of breast cancer types can be associated with tumors arising from different epithelial cells in the hierarchical tree, suggesting that multiple cell types have the capacity to become tumorigenic (Visvader 2009). This may be similar in prostate cancer, but our understanding is not so advanced as in breast cancer and based on the occurrence of luminal or neuroendocrine tumors, subclassifications of tumor linked to the specific cell type of origin are unknown.

There is evidence to implicate both basal and luminal populations containing cancer cell(s) of origin in prostate cancer. First, multiple lines of evidence

demonstrate initiation of prostate cancer from luminal (and possibly intermediate) cells, based on targeted gene disruption by Cre-recombinase under the control of the probasin (either probasin or ARR$_2$/probasin) or PSA promoters that show luminal-specific expression. The genetic targets vary widely, including *Pten* tumor suppressor gene (Ma *et al*. 2005), myc (Ellwood-Yen *et al*. 2003), and *Nkx3-1* (Iwata *et al*. 2010), which result in tumorigenesis under luminal-specific expression. Targeted deletion of *Pten* in CARNs results in rapid carcinoma formation after androgen-mediated regeneration (Wang *et al*. 2009). These data indicate that luminal cells, including the CARNs as luminal stem cells, represent a population that is an efficient target for oncogenic transformation in prostate cancer. Together, these findings indicate that luminal cells represent a potential cancer cell of origin.

Alternatively, a body of work by the Witte Laboratory provides evidence to suggest that basal cells can initiate preneoplastic and cancerous lesions. For example, loss of *PTEN* negatively regulates *p63*$^+$ prostatic basal cell proliferation without blocking differentiation, resulting in an expansion of a prostate stem/progenitor-like subpopulation, using defined cell lineage markers including Sca-1$^+$, Bcl-2$^+$, and CD49f^{hi} cells (Wang *et al*. 2006, Mulholland *et al*. 2009, Lawson *et al*. 2010). Most recently, this group introduced a series of genetic alterations into prospectively identified populations of mouse basal stem and luminal cells and showed basal cells, but not luminal cells, are efficient targets for prostate cancer initiation (Lawson *et al*. 2010). Whether there are two independent cancer cells of origin in the prostate remains unknown.

When considering translation of this information to human tissues, again the data are limited. Studies in human prostate cancer indicate that CD133$^+$ is a common marker of cells with stem-cell-like properties in both nonmalignant and malignant tissues (Richardson *et al*. 2004, Collins *et al*. 2005). This led to the assumption that CD133$^+$ stem cells in normal tissues are the cancer cell of origin in prostate cancer. However, an opposing view was proposed by Vander Griend *et al*. (2008), who proposed that prostate cancer is derived from AR$^+$ cells, which are unlikely to be stem cells in normal tissues, and tumor cells subsequently acquire 'stem-like activity' by gaining expression of CD133$^+$. Similar to breast cancer, there may be more than one cellular origin of human prostate cancer, which may correlate with tumor phenotype, but is yet to be formally documented.

Cancer-repopulating cells

In cancer, a population of biologically distinct tumor cells possessing stem cell properties are defined as CRCs. These cells have proliferative potential to maintain tumor bulk and resist chemotherapy in order to repopulate the tumor and cause metastasis after cancer treatment (Reya *et al*. 2001). In general, CRCs are proposed to conform to one of two proposed models of differentiation. Originally, a hierarchical model of CRC differentiation suggests that CRCs and their progenitors give rise to more differentiated cells with less regenerative potential (Reya *et al*. 2001, Dick 2009). This model is based on fractionation of tumor cells using cell surface markers to isolate rare subsets of tumors cells from the brain, blood, and colon that display exclusive tumor regenerating potential in colony-forming assays and *in vivo* transplantations in immune-deficient mice (Bonnet & Dick 1997, O'Brien *et al*. 2007). However, xenotransplantation studies using fractionated cell populations are complicated because of the tumor cell interactions with the microenvironment, mediated by both soluble and membrane-bound factors (Hanahan & Weinberg 2000). The rarity of human tumor cells that survive transplantation may simply reflect the cells that can most readily adapt to growth in a foreign (mouse) milieu. This is confirmed by the variation in results with advancing use of different immune compromised mouse hosts, such as NOD-SCID-IL2Rγ^{null} (NSG) mice (Kelly *et al*. 2007). In melanoma, the original frequency of xenotransplantation of human metastatic melanoma cells is reported to be 1 in 1 090 000 when transplanted into NOD/SCID mice (Schatton *et al*. 2008), whereas recent data by Quintana *et al*. (2008) showed that ∼1 in 4 (25%) unselected tumor cells are capable of tumor formation in NSG mice, demonstrating the crucial reliance of optimal transplantation conditions in determining tumorigenic potential *in vivo*. Therefore, tumorigenic assays for prostate CRCs must ensure the survival of human cancer cells in the model being used, so that any read out of repopulating potential is related only to its biological properties, and not to limitations of the assay. Based on the repopulating capacity of a greater proportion of the tumor in NSG mice, an alternative CRC model is proposed, where all cancer cells are homogeneous (equal), and that random influences change the behavior of individual cells, including intrinsic factors such as transcription factors or signaling pathways and/or extrinsic factors such as host factors, microenvironment, and immune

response. This stochastic model, based on clonal evolution, suggests that CRCs are derived from populations of cancer cells that confer a selective growth advantage and are not restricted to a particular cell type within the tumor (Campbell & Polyak 2007). Unlike the hierarchical model, clonal evolution is a nonstructured multistaged process, where different clones can obtain this advantage throughout the cancer progression, resulting in intratumoral variation (Shackleton *et al.* 2009). This alternate model also provides a plausible explanation for the biological and functional heterogeneity detected in tumors.
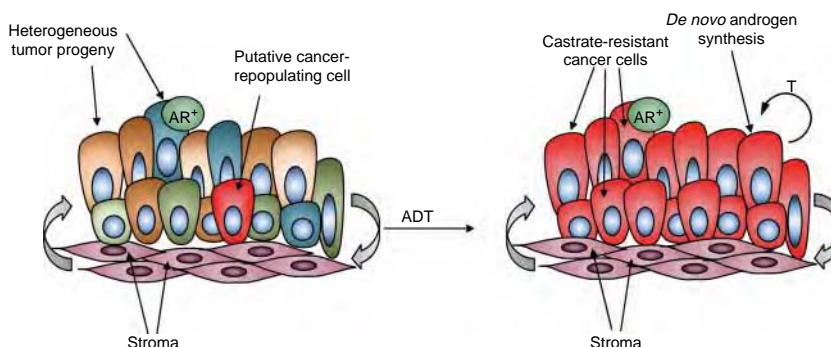
CRCs in prostate cancer

In prostate cancer, the identification and characterization of CRCs (or cells with selective growth advantage) may be different in androgen-dependent disease compared to castrate-resistant disease. Therefore, in this section, we will consider localized and CRPC separately (Fig. 3). Localized tumors are composed of a heterogeneous mix of cell types, and the differentiation capacity and hierarchical relationship between these cell types have not been defined, particularly in human tissues. Less is known about the cellular components of CRPC tumors. As mice are extremely resistant to prostate cancer initiation, models of carcinogenesis in rodents are often artificial and show minimal resemblance to the actual biology in human prostate. Therefore, the majority of studies that attempt to identity and characterize CRCs in prostate cancer use human tissues.

CRCs in localized prostate cancer

The identity of CRCs in prostate cancer, defined by the functional ability to undergo self-renewal and differentiate the entire progeny of the tumor mass, is unclear. The putative stem cell marker, $CD133^+$, isolates prostate cancer cells with stem-cell-like properties, including a significant capacity for self-renewal and regeneration of phenotypically mixed populations of nonclonogenic cells that express differentiated cell products, including AR and PSA *in vitro* (Collins *et al.* 2005). This population represents $\sim 0.1\%$ of cells in prostatic tumors, without correlation to Gleason grade. These data, conducted using *in vitro* assays, represent the only attempt to prospectively isolate CRCs in human prostate cancer. Unlike other tumor types, including melanoma, leukemia, brain, or colon, functional identification of CRCs using transplantation and limiting dilution assays *in vivo* has not been conducted. This is an important area of research if we are to consider CRCs as cellular or molecular targets for therapy.

CRCs in CRPC

Without identifying or characterizing CRCs in localized prostate cancer, it is difficult to determine their role in progression to metastasis or the development of CRPC. Regardless, it is evident that there is a subpopulation of prostate cancer cells that are resistant to current therapeutics, particularly ADT, which is a front line therapy for advanced disease. When tumors relapse after ADT, presumably because of the repopulating potential of a subset of cancer cells, treatment consists of symptom management and at

Figure 3 Prostate cancer-repopulating cells. In localized, androgen-dependent prostate cancer (left panel), cancer-repopulating cells (CRCs) are proposed to be a rare subpopulation distinguishable from the bulk of the tumor by their ability to survive treatment and regenerate tumor mass. The identity of these cells is less defined than that of in other solid tumors, but CD133 is postulated to enrich prostate cancer CRCs. The expression status of AR in human $CD133^+$ CRCs remains under debate, but the tumor bulk is AR^+ and is therefore androgen dependent. After failed front line therapies (i.e. radical prostatectomy or radiotherapy), patients commonly undergo androgen deprivation therapy (ADT). Recurrent disease after this treatment leads to castrate-resistant prostate cancer. In these tumors, the residual cancer cells gain the ability to adapt the androgen-depleted environment and synthesize their own androgens *de novo* in order to mediate and maintain cancer cell survival and growth. It is unknown whether the adaptive ability is common to all cancer cells or restricted to CRCs from the earlier stage tumor.

times toxic chemotherapy although patients invariably succumb to the disease.

In CRPC, tumor cells adapt to the low-androgen environment and continue to mediate androgen signaling by AR overexpression, amplification, mutation, and altered coregulator interactions (Scher *et al*. 2004), but also by gaining the ability to synthesize sufficient androgens *de novo* to activate AR pathways and allow the growth of cancer despite negligible amounts of androgens in the circulation (Locke *et al*. 2008; Fig. 3). Whether all cells or only a selected population are capable of renewing and repopulating based on their ability to make androgens or express the AR (potentially as a result of clonal selection) is unknown. Identifying the subpopulation responsible for *de novo* steroid genesis would give invaluable insight into advanced prostate cancer biology, and create novel targets for castrate-resistant disease.

Molecular targets for CRCs in prostate cancer

In an attempt to identify molecular factors that can specifically target stem cells in normal tissue or CRCs in prostate cancer, two genetic profiling studies are reported on benign and malignant prostatic CD133$^+$ epithelial cells isolated from human specimens. In benign prostatic hyperplasia, CD133$^+$ cells expressed genes relating to undifferentiated cells such as TDGF1, and targets of the Wnt and Hedgehog developmental pathways, whereas CD133$^-$ cells showed upregulated proliferation and metabolism genes, related more specifically to a transient amplifying population (Shepherd *et al*. 2008). In cancer, specifically CRPC, CD133$^+$ cells displayed a more transient amplifying population phenotype with increased metabolic activity and proliferation, possibly explaining the transition from a relatively quiescent state to an active growing tumor phenotype, perhaps reflecting that CD133 isolates biologically distinct cells from benign compared to malignant tissues (Shepherd *et al*. 2008). Similar array analysis on cultured samples of localized primary human prostate cancer reveals CD133$^+$ cells display a pro-inflammatory phenotype as NFκβ expression is increased reflecting the immune responsiveness of CD133$^+$ cells (Birnie *et al*. 2008). With further investigation and discovery of the identity of CRCs, the molecular targets within these cells will become evident and may lead to clinical applications for men with prostate cancer.

The role of endocrine hormones in regulation of stem cell types

All three stem cells (normal stem cells, cancer cell of origin, and CRCs) reside in a niche environment, predominantly composed of prostatic stroma that plays a major role in dictating stem cell fate. Extensive studies on prostate gland development show that epithelial differentiation is induced and maintained by stromal signaling, specifically mediated by hormonal and paracrine signaling mechanisms (Cunha & Donjacour 1989); direct androgen binding to epithelial ARs is not required for epithelial differentiation (Chung & Cunha 1983, Takeda *et al*. 1990), but is essential for the induction and maintenance of secretory activity (Donjacour & Cunha 1993, Cunha 1994). The potent effect of stromal induction and essential requirement of androgens for prostate development are reflected by the fact that other tissue types differentiate into prostate when grown with inductive prostatic mesenchyme in male host mice that provide an androgen-rich endocrine environment (Taylor *et al*. 2009). Of note, almost all the *in vivo* studies testing the growth and/or tumor potential of subpopulations of cells, co-transplanted inductive prostatic stroma (Lawson *et al*. 2007, 2010, Goldstein *et al*. 2008, Leong *et al*. 2008, Mulholland *et al*. 2009, Lukacs *et al*. 2010). Without the stromal-mediated AR signaling, it is unlikely that the fractionated cell populations would survive and/or proliferate *in vivo*, regardless of their AR status, as stromal–epithelial signaling is integral to prostatic differentiation.

In prostate cancer, the stromal niche or micro-environment also plays a critical role in regulating differentiation of CRCs, potentially by altered endocrine and/or paracrine signaling. Prostatic tumor stroma has a distinct phenotype that is known to facilitate tumorigenesis (Taylor & Risbridger 2008), whereby carcinoma-associated fibroblasts can promote tumor progression and contribute to metastasis (Olumi *et al*. 1999). Some of the key regulators of this activity are members of the transforming growth factor β superfamily and/or specific chemokines and cytokines that promote malignant transformation of the epithelium (Joesting *et al*. 2005, Ao *et al*. 2007). Although AR expression is high in developing nonmalignant prostatic stroma, AR expression in prostate cancer stroma is often low to detect (Henshall *et al*. 2001, Ricciardelli *et al*. 2005, Wikstrom *et al*. 2009). This provides an imbalance in the stromal–epithelial steroid signaling in cancer compared to normal tissues, and this altered microenvironment significantly affects the growth and differentiation signals received by the

epithelium, especially CRCs, although this interaction remains relatively unexplored.

Similar to the prostate gland, the mammary gland is a hormone-dependent organ susceptible to tumorigenesis. The recent prospective isolation of MaSCs (in addition to committed progenitor and mature luminal cells) from murine tissues shows a receptor-negative phenotype for ERα, PR, and ErbB2 (Asselin-Labat *et al.* 2006, Shackleton *et al.* 2006). Regardless of this, MaSCs are highly responsive to steroid hormone signaling; ovariectomy markedly diminished MaSC number and outgrowth potential *in vivo*, whereas estrogen and progesterone increased MaSC activity in mice (Asselin-Labat *et al.* 2010). The same is likely to be true for prostatic stem cells, but has not been tested.

Without a clearly defined stem cell population in normal or cancerous prostate tissues, the steroid receptor status and responsiveness to androgens remain undefined. Prostatic basal cells demonstrate low levels of AR but are androgen responsive, as demonstrated by regeneration of the prostatic tissue from basal cells during re-administration of testosterone after castration in rodents (Montpetit *et al.* 1988, Verhagen *et al.* 1988, Bonkhoff & Remberger 1993, De Marzo *et al.* 1998). Basal cells that show stem cell properties, based on Sca-1$^+$ cells in mouse and CD133$^+$ cells in human, also express low or no AR expression compared to their negative counterparts (Wang *et al.* 2006, Heer *et al.* 2007). The AR expression and sensitivity to androgens is characterized in the linear hierarchical model where CK5$^+$8$^-$ basal cells (containing stem cells and transient amplifying or progenitor cells) are AR$^-$, whereas CK5$^+$8$^+$ intermediate and CK5$^-$8$^+$ luminal cells are AR$^+$ (De Marzo *et al.* 1998, Uzgare *et al.* 2004). In keeping with known expression patterns of luminal prostatic epithelia, CARNs (luminal stem cells) always express AR (Wang *et al.* 2009). In human prostate cancer, there is a debate over the AR status of CD133$^+$ cells, as they were originally reported to be AR$^-$ (Richardson *et al.* 2004), but conflicting data suggest that CD133$^+$ cells responsible for tumor propagation and progression are AR$^+$ and therefore are direct targets for androgen stimulation (Vander Griend *et al.* 2008). As the use of cell surface markers to isolate fractionated cell populations usually enriches for cells of mixed phenotypes, the function and repopulating potential of AR$^+$ and/or AR$^-$ cells remains unknown. It is possible with the advancing identification of prostatic stem cells that a pure AR$^-$ population will be identified in nonmalignant tissues, similar to the MaSC, but the AR status in localized or CRPC tumors may vary between cancer types. Additionally, the expression of estrogen receptors

(ERs), ERα and ERβ is unknown, but is of interest based on the integral role of estrogens in prostate carcinogenesis (Ellem & Risbridger 2007, Risbridger *et al.* 2010). Overall, ERα is low to detect in prostatic epithelial cells where ERβ is predominantly expressed. ERβ expression is highly variable in human tissues depending on disease status (Leav *et al.* 2001). Recent data from our laboratory show that an ERβ agonist compound selectively induces apoptosis in castrate-resistant CD133$^+$ basal cells, providing a rationale for further exploration of the role of ERβ in prostatic stem cells and in cancer (McPherson *et al.* 2010).

Conclusions

Relapse of prostate cancer in patients with advanced disease after ADT occurs because a proportion of cancer cells resist hormone therapy and become castrate resistant. It is possible that these cells are CRCs that we believe should continue to be the focus of intensive research effort. Achieving this goal is hampered by lack of clarity around the identity of the normal prostatic stem cell(s) and the cancer cell(s) of origin. Stem cells exist in both basal and luminal cell populations and are postulated to comprise intermediate cells that possess characteristics of both cell types. Likewise, the cellular origin of cancer can be from basal and/or luminal cells, using mouse models of prostate cancer. Although we continue to search for the identity of specific stem cells types (normal prostatic stem cells, cancer cells of origin, and/or CRCs) that we believe reside in the normal or diseased epithelium, it is entirely possible that 'stemness' is acquired during repair or tumor propagation and should be considered as a dynamic or transient process that evolves due to environmental pressures, rather than an inherent or sustained property of particular cell types. This creates many challenges in stem cell research as changes in environment, which occur in cell culture and animal models, may suppress the stem cell phenotype that is active within the tumor. The development of models that accurately recreate the tumor microenvironment are vital in order to more accurately assess treatment strategies for their ability to destroy stem cells rather than just decrease the overall tumor size. By using this strategy it is hoped that treatments can be developed to rid patients of the tumors cells responsible for disease relapse and to cure them of their disease.

In the absence of agreement on the identities of cancer cells of origin or CRCs, their androgen sensitivity and responsiveness remains equivocal. The contribution of stroma to stem cell differentiation is also rarely considered, although it clearly plays

an important role in mediating androgen-stimulated paracrine signaling from the stromal microenvironment. The initial hype and hope surrounding CRCs remains, but relies on the identification of these cell populations in localized and advanced disease.

Declaration of interest

Funding

Author contribution statement

All authors contributed to the manuscript and figures.

Acknowledgments

References

Abrahamsson PA 1999 Neuroendocrine cells in tumour growth of the prostate. *Endocrine-Related Cancer* **6** 503–519. (doi:10.1677/erc.0.0060503)

Ao M, Franco OE, Park D, Raman D, Williams K & Hayward SW 2007 Cross-talk between paracrine-acting cytokine and chemokine pathways promotes malignancy in benign human prostatic epithelium. *Cancer Research* **67** 4244–4253. (doi:10.1158/0008-5472.CAN-06-3946)

Asselin-Labat ML, Shackleton M, Stingl J, Vaillant F, Forrest NC, Eaves CJ, Visvader JE & Lindeman GJ 2006 Steroid hormone receptor status of mouse mammary stem cells. *Journal of the National Cancer Institute* **98** 1011–1014. (doi:10.1093/jnci/djj267)

Asselin-Labat ML, Vaillant F, Sheridan JM, Pal B, Wu D, Simpson ER, Yasuda H, Smyth GK, Martin TJ, Lindeman GJ *et al.* 2010 Control of mammary stem cell function by steroid hormone signalling. *Nature* **465** 798–802. (doi:10.1038/nature09027)

Birnie R, Bryce SD, Roome C, Dussupt V, Droop A, Lang SH, Berry PA, Hyde CF, Lewis JL, Stower MJ *et al.* 2008 Gene expression profiling of human prostate cancer stem cells reveals a pro-inflammatory phenotype and the importance of extracellular matrix interactions. *Genome Biology* **9** R83. (doi:10.1186/gb-2008-9-5-r83)

Bonkhoff H 1996 Role of the basal cells in premalignant changes of the human prostate: a stem cell concept for the development of prostate cancer. *European Urology* **30** 201–205.

Bonkhoff H & Remberger K 1993 Widespread distribution of nuclear androgen receptors in the basal cell layer of the normal and hyperplastic human prostate. *Virchows Archiv. A, Pathological Anatomy and Histopathology* **422** 35–38. (doi:10.1007/BF01605130)

Bonkhoff H, Stein U & Remberger K 1994 The proliferative function of basal cells in the normal and hyperplastic human prostate. *Prostate* **24** 114–118. (doi:10.1002/pros.2990240303)

Bonnet D & Dick JE 1997 Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Medicine* **3** 730–737. (doi:10.1038/nm0797-730)

Bostwick D & Dundore P 1997 Normal anatomy and histology. In *Biopsy Pathology of the Prostate*, 1st edn, pp 1–26. Eds AM Neville, F Walker, CR Taylor & LS Gottlieb. London, UK: Chapman & Hall.

Burger PE, Xiong X, Coetzee S, Salm SN, Moscatelli D, Goto K & Wilson EL 2005 Sca-1 expression identifies stem cells in the proximal region of prostatic ducts with high capacity to reconstitute prostatic tissue. *PNAS* **102** 7180–7185. (doi:10.1073/pnas.0502761102)

Campbell LL & Polyak K 2007 Breast tumor heterogeneity: cancer stem cells or clonal evolution? *Cell Cycle* **6** 2332–2338.

Chung LW & Cunha GR 1983 Stromal–epithelial interactions: II. Regulation of prostatic growth by embryonic urogenital sinus mesenchyme. *Prostate* **4** 503–511. (doi:10.1002/pros.2990040509)

Coffey DS 1992 The molecular biology, endocrinology, and physiology of the prostate and seminal vesicles. In *Campbell's Urology*, 6th edn, pp 221–301. Eds PC Walsh, AB Retik, TA Stamey & ED Vaughan. Philadelphia PA, USA: WB Saunders.

Collins AT, Habib FK, Maitland NJ & Neal DE 2001 Identification and isolation of human prostate epithelial stem cells based on alpha(2)beta(1)-integrin expression. *Journal of Cell Science* **114** 3865–3872.

Collins AT, Berry PA, Hyde C, Stower MJ & Maitland NJ 2005 Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Research* **65** 10946–10951. (doi:10.1158/0008-5472.CAN-05-2018)

Cunha AR 1994 Role of mesenchymal–epthelial interactions in normal and abnormal development of male urogenital glands. In *Ultrastructure of Male Urogenital Glands: Prostate, Seminal Vesicles, Urethral, and Bulbourethral Glands*, Eds A Riva, F Testa Riva & PM Motta. Boston, MA, USA: Kluwer Academic Publishers.

Cunha GR & Donjacour AA 1989 Mesenchymal–epithelial interactions in the growth and development of the prostate. *Cancer Treatment and Research* **46** 159–175.

De Marzo AM, Meeker AK, Epstein JI & Coffey DS 1998 Prostate stem cell compartments: expression of the cell cycle inhibitor p27Kip1 in normal, hyperplastic, and neoplastic cells. *American Journal of Pathology* **153** 911–919.

DeKlerk DP & Coffey DS 1978 Quantitative determination of prostatic epithelial and stromal hyperplasia by a new technique. Biomorphometrics. *Investigative Urology* **16** 240–245.

Dick JE 2009 Looking ahead in cancer stem cell research. *Nature Biotechnology* **27** 44–46. (doi:10.1038/nbt0109-44)

Donjacour A & Cunha G 1993 Assessment of prostatic protein secretion in tissue recombinants made of urogenital sinus mesenchyme and urothelium from normal or androgen-insensitive mice. *Endocrinology* **132** 2342–2350. (doi:10.1210/en.132.6.2342)

El-Alfy M, Pelletier G, Hermo LS & Labrie F 2000 Unique features of the basal cells of human prostate epithelium. *Microscopy Research and Technique* **51** 436–446. (doi:10.1002/1097-0029(20001201)51:5<436::AID-JEMT6>3.0.CO;2-T)

Ellem SJ & Risbridger GP 2007 Treating prostate cancer: a rationale for targeting local oestrogens. *Nature Reviews. Cancer* **7** 621–627. (doi:10.1038/nrc2174)

Ellwood-Yen K, Graeber TG, Wongvipat J, Iruela-Arispe ML, Zhang J, Matusik R, Thomas GV & Sawyers CL 2003 Myc-driven murine prostate cancer shares molecular features with human prostate tumors. *Cancer Cell* **4** 223–238. (doi:10.1016/S1535-6108(03)00197-1)

Foster CS, Dodson A, Karavana V, Smith PH & Ke Y 2002 Prostatic stem cells. *Journal of Pathology* **197** 551–565. (doi:10.1002/path.1194)

Goldstein AS, Lawson DA, Cheng D, Sun W, Garraway IP & Witte ON 2008 Trop2 identifies a subpopulation of murine and human prostate basal cells with stem cell characteristics. *PNAS* **105** 20882–20887. (doi:10.1073/pnas.0811411106)

Grisanzio C & Signoretti S 2008 p63 in prostate biology and pathology. *Journal of Cellular Biochemistry* **103** 1354–1368. (doi:10.1002/jcb.21555)

Hanahan D & Weinberg RA 2000 The hallmarks of cancer. *Cell* **100** 57–70. (doi:10.1016/S0092-8674(00)81683-9)

Hayward SW, Rosen MA & Cunha GR 1997 Stromal–epithelial interactions in the normal and neoplastic prostate. *British Journal of Urology* **79** (Suppl 2) 18–26.

Heer R, Robson CN, Shenton BK & Leung HY 2007 The role of androgen in determining differentiation and regulation of androgen receptor expression in the human prostatic epithelium transient amplifying population. *Journal of Cellular Physiology* **212** 572–578. (doi:10.1002/jcp.21154)

Henshall SM, Quinn DI, Lee CS, Head DR, Golovsky D, Brenner PC, Delprado W, Stricker PD, Grygiel JJ & Sutherland RL 2001 Altered expression of androgen receptor in the malignant epithelium and adjacent stroma is associated with early relapse in prostate cancer. *Cancer Research* **61** 423–427.

Hudson DL 2004 Epithelial stem cells in human prostate growth and disease. *Prostate Cancer and Prostatic Diseases* **7** 188–194. (doi:10.1038/sj.pcan.4500745)

Humphrey PA 2007 Diagnosis of adenocarcinoma in prostate needle biopsy tissue. *Journal of Clinical Pathology* **60** 35–42. (doi:10.1136/jcp.2005.036442)

Isaacs JT & Coffey DS 1989 Etiology and disease process of benign prostatic hyperplasia. *Prostate. Supplement* **2** 33–50. (doi:10.1002/pros.2990150506)

Iwata T, Schultz D, Hicks J, Hubbard GK, Mutton LN, Lotan TL, Bethel C, Lotz MT, Yegnasubramanian S, Nelson WG et al. 2010 MYC overexpression induces prostatic intraepithelial neoplasia and loss of Nkx3.1 in mouse luminal epithelial cells. *PLoS ONE* **5** e9427. (doi:10.1371/journal.pone.0009427)

Joesting MS, Perrin S, Elenbaas B, Fawell SE, Rubin JS, Franco OE, Hayward SW, Cunha GR & Marker PC 2005 Identification of SFRP1 as a candidate mediator of stromal-to-epithelial signaling in prostate cancer. *Cancer Research* **65** 10423–10430. (doi:10.1158/0008-5472.CAN-05-0824)

Kelly PN, Dakic A, Adams JM, Nutt SL & Strasser A 2007 Tumor growth need not be driven by rare cancer stem cells. *Science* **317** 337. (doi:10.1126/science.1142596)

Kurita T, Medina RT, Mills AA & Cunha GR 2004 Role of p63 and basal cells in the prostate. *Development* **131** 4955–4964. (doi:10.1242/dev.01384)

Kyprianou N & Isaacs JT 1988 Identification of a cellular receptor for transforming growth factor-beta in rat ventral prostate and its negative regulation by androgens. *Endocrinology* **123** 2124–2131. (doi:10.1210/endo-123-4-2124)

Lawson DA, Xin L, Lukacs RU, Cheng D & Witte ON 2007 Isolation and functional characterization of murine prostate stem cells. *PNAS* **104** 181–186. (doi:10.1073/pnas.0609684104)

Lawson DA, Zong Y, Memarzadeh S, Xin L, Huang J & Witte ON 2010 Basal epithelial stem cells are efficient targets for prostate cancer initiation. *PNAS* **107** 2610–2615. (doi:10.1073/pnas.0913873107)

Leav I, Lau KM, Adams JY, McNeal JE, Taplin ME, Wang J, Singh H & Ho SM 2001 Comparative studies of the estrogen receptors beta and alpha and the androgen receptor in normal human prostate glands, dysplasia, and in primary and metastatic carcinoma. *American Journal of Pathology* **159** 79–92.

van Leenders GJ & Schalken JA 2001 Stem cell differentiation within the human prostate epithelium: implications for prostate carcinogenesis. *BJU International* **88** (Suppl 2) 35–42 (discussion 49–50). (doi:10.1046/j.1464-410X.2001.00117.x)

Leong KG, Wang BE, Johnson L & Gao WQ 2008 Generation of a prostate from a single adult stem cell. *Nature* **456** 804–808. (doi:10.1038/nature07427)

Lim E, Vaillant F, Wu D, Forrest NC, Pal B, Hart AH, Asselin-Labat ML, Gyorki DE, Ward T, Partanen A et al. 2009 Aberrant luminal progenitors as the candidate

target population for basal tumor development in BRCA1 mutation carriers. *Nature Medicine* **15** 907–913. (doi:10.1038/nm.2000)

Liu AY, True LD, LaTray L, Nelson PS, Ellis WJ, Vessella RL, Lange PH, Hood L & van den Engh G 1997 Cell–cell interaction in prostate gene regulation and cytodifferentiation. *PNAS* **94** 10705–10710. (doi:10.1073/pnas.94.20.10705)

Locke JA, Guns ES, Lubik AA, Adomat HH, Hendy SC, Wood CA, Ettinger SL, Gleave ME & Nelson CC 2008 Androgen levels increase by intratumoral *de novo* steroidogenesis during progression of castration-resistant prostate cancer. *Cancer Research* **68** 6407–6415. (doi:10.1158/0008-5472.CAN-07-5997)

Lukacs RU, Goldstein AS, Lawson DA, Cheng D & Witte ON 2010 Isolation, cultivation and characterization of adult murine prostate stem cells. *Nature Protocols* **5** 702–713. (doi:10.1038/nprot.2010.11)

Ma X, Ziel-van der Made AC, Autar B, van der Korput HA, Vermeij M, van Duijn P, Cleutjens KB, de Krijger R, Krimpenfort P, Berns A *et al.* 2005 Targeted biallelic inactivation of Pten in the mouse prostate leads to prostate cancer accompanied by increased epithelial cell proliferation but not by reduced apoptosis. *Cancer Research* **65** 5730–5739. (doi:10.1158/0008-5472.CAN-04-4519)

McNeal JE 1981 Normal and pathologic anatomy of prostate. *Urology* **17** 11–16.

McNeal JE 1988 Normal histology of the prostate. *American Journal of Surgical Pathology* **12** 619–633. (doi:10.1097/00000478-198808000-00003)

McPherson SJ, Hussain S, Balanathan P, Hedwards S, Niranjan B, Grant M, Chandrasiri UP, Toivanen R, Wang Y, Taylor RA *et al.* 2010 Estrogen receptor beta activated apoptosis in benign hyperplasia and cancer of the prostate is androgen-independent and TNF-A mediated. *PNAS* **107** 3123–3128. (doi:10.1073/pnas.0905524107)

Montpetit M, Abrahams P, Clark AF & Tenniswood M 1988 Androgen-independent epithelial cells of the rat ventral prostate. *Prostate* **12** 13–28. (doi:10.1002/pros.2990120104)

Mulholland DJ, Xin L, Morim A, Lawson D, Witte O & Wu H 2009 Lin-Sca-1+CD49f high stem/progenitors are tumor-initiating cells in the Pten-null prostate cancer model. *Cancer Research* **69** 8555–8562. (doi:10.1158/0008-5472.CAN-08-4673)

O'Brien CA, Pollett A, Gallinger S & Dick JE 2007 A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* **445** 106–110. (doi:10.1038/nature05372)

Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD & Cunha GR 1999 Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Research* **59** 5002–5011.

Penson D & Chan J 2007 Urological diseases in America project: Prostate cancer. *Journal of Urology* **177** 2020–2029. (doi:10.1016/j.juro.2007.01.121)

Potten CS & Loeffler M 1990 Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development* **110** 1001–1020.

Quintana E, Shackleton M, Sabel MS, Fullen DR, Johnson TM & Morrison SJ 2008 Efficient tumour formation by single human melanoma cells. *Nature* **456** 593–598. (doi:10.1038/nature07567)

Reya T, Morrison SJ, Clarke MF & Weissman IL 2001 Stem cells, cancer, and cancer stem cells. *Nature* **414** 105–111. (doi:10.1038/35102167)

Ricciardelli C, Choong CS, Buchanan G, Vivekanandan S, Neufing P, Stahl J, Marshall VR, Horsfall DJ & Tilley WD 2005 Androgen receptor levels in prostate cancer epithelial and peritumoral stromal cells identify non-organ confined disease. *Prostate* **63** 19–28. (doi:10.1002/pros.20154)

Richardson GD, Robson CN, Lang SH, Neal DE, Maitland NJ & Collins AT 2004 CD133, a novel marker for human prostatic epithelial stem cells. *Journal of Cell Science* **117** 3539–3545. (doi:10.1242/jcs.01222)

Risbridger GP, Davis ID, Birrell SN & Tilley W 2010 Breast and prostate cancer: more similar than different. *Nature Reviews. Cancer* **10** 205–212. (doi:10.1038/nrc2795)

Schatton T, Murphy GF, Frank NY, Yamaura K, Waaga-Gasser AM, Gasser M, Zhan Q, Jordan S, Duncan LM, Weishaupt C *et al.* 2008 Identification of cells initiating human melanomas. *Nature* **451** 345–349. (doi:10.1038/nature06489)

Scher HI, Buchanan G, Gerald W, Butler LM & Tilley WD 2004 Targeting the androgen receptor: improving outcomes for castration-resistant prostate cancer. *Endocrine-Related Cancer* **11** 459–476. (doi:10.1677/erc.1.00525)

Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, Wu L, Lindeman GJ & Visvader JE 2006 Generation of a functional mammary gland from a single stem cell. *Nature* **439** 84–88. (doi:10.1038/nature04372)

Shackleton M, Quintana E, Fearon ER & Morrison SJ 2009 Heterogeneity in cancer: cancer stem cells versus clonal evolution. *Cell* **138** 822–829. (doi:10.1016/j.cell.2009.08.017)

Shepherd CJ, Rizzo S, Ledaki I, Davies M, Brewer D, Attard G, de Bono J & Hudson DL 2008 Expression profiling of CD133+ and CD133− epithelial cells from human prostate. *Prostate* **68** 1007–1024. (doi:10.1002/pros.20765)

Signoretti S & Loda M 2007 Prostate stem cells: from development to cancer. *Seminars in Cancer Biology* **17** 219–224. (doi:10.1016/j.semcancer.2006.04.004)

Signoretti S, Waltregny D, Dilks J, Isaac B, Lin D, Garraway L, Yang A, Montironi R, McKeon F & Loda M 2000 p63 is a prostate basal cell marker and is required for prostate development. *American Journal of Pathology* **157** 1769–1775.

Takeda H, Suematsu N & Mizuno T 1990 Transcription of prostatic steroid binding protein (PSBP) gene is induced by epithelial–mesenchymal interaction. *Development* **110** 273–281.

Taylor RA & Risbridger GP 2008 Prostatic tumor stroma: a key player in cancer progression. *Current Cancer Drug Targets* **8** 490–497. (doi:10.2174/156800908785699351)

Taylor RA, Wang H, Wilkinson SE, Richards MG, Britt KL, Vaillant F, Lindeman GJ, Visvader JE, Cunha GR, St John J *et al.* 2009 Lineage enforcement by inductive mesenchyme on adult epithelial stem cells across developmental germ layers. *Stem Cells* **27** 3032–3042. (doi:10.1002/stem.244)

Tsujimura A, Fujita K, Komori K, Takao T, Miyagawa Y, Takada S, Matsumiya K, Nonomur N & Okuyama A 2007 Prostatic stem cell marker identified by cDNA microarray in mouse. *Journal of Urology* **178** 686–691. (doi:10.1016/j.juro.2007.03.092)

Uzgare AR, Xu Y & Isaacs JT 2004 *In vitro* culturing and characteristics of transit amplifying epithelial cells from human prostate tissue. *Journal of Cellular Biochemistry* **91** 196–205. (doi:10.1002/jcb.10764)

Vander Griend DJ, Karthaus WL, Dalrymple S, Meeker A, DeMarzo AM & Isaacs JT 2008 The role of CD133 in normal human prostate stem cells and malignant cancer-initiating cells. *Cancer Research* **68** 9703–9711. (doi:10.1158/0008-5472.CAN-08-3084)

Verhagen AP, Aalders TW, Ramaekers FC, Debruyne FM & Schalken JA 1988 Differential expression of keratins in the basal and luminal compartments of rat prostatic epithelium during degeneration and regeneration. *Prostate* **13** 25–38. (doi:10.1002/pros.2990130104)

Visvader JE 2009 Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. *Genes and Development* **23** 2563–2577. (doi:10.1101/gad.1849509)

Wang Y, Hayward S, Cao M, Thayer K & Cunha G 2001 Cell differentiation lineage in the prostate. *Differentiation* **68** 270–279. (doi:10.1046/j.1432-0436.2001.680414.x)

Wang S, Garcia AJ, Wu M, Lawson DA, Witte ON & Wu H 2006 Pten deletion leads to the expansion of a prostatic stem/progenitor cell subpopulation and tumor initiation. *PNAS* **103** 1480–1485. (doi:10.1073/pnas.0510652103)

Wang X, Kruithof-de Julio M, Economides KD, Walker D, Yu H, Halili MV, Hu YP, Price SM, Abate-Shen C & Shen MM 2009 A luminal epithelial stem cell that is a cell of origin for prostate cancer. *Nature* **461** 495–500. (doi:10.1038/nature08361)

Watt FM & Hogan BL 2000 Out of Eden: stem cells and their niches. *Science* **287** 1427–1430. (doi:10.1126/science.287.5457.1427)

Wicha MS, Liu S & Dontu G 2006 Cancer stem cells: an old idea – a paradigm shift. *Cancer Research* **66** 1883–1890 (discussion 1895–1886). (doi:10.1158/0008-5472.CAN-05-3153)

Wikstrom P, Marusic J, Stattin P & Bergh A 2009 Low stroma androgen receptor level in normal and tumor prostate tissue is related to poor outcome in prostate cancer patients. *Prostate* **69** 799–809. (doi:10.1002/pros.20927)

Xin L, Lawson DA & Witte ON 2005 The Sca-1 cell surface marker enriches for a prostate-regenerating cell subpopulation that can initiate prostate tumorigenesis. *PNAS* **102** 6942–6947. (doi:10.1073/pnas.0502320102)

Yao M, Taylor RA, Richards MG, Sved P, Wong J, Eisinger D, Xie C, Salomon R, Risbridger GP & Dong Q 2010 Prostate-regenerating capacity of cultured human adult prostate epithelial cells. *Cells, Tissues, Organs* **191** 203–212. (doi:10.1159/000240244)